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Trichoderma harzianum: Inhibition of mycotoxin producing fungi and toxin biosynthesis

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ABSTRACT

A quarter of the world-wide crop is spoiled by filamentous fungi and their mycotoxins and weather extremes associated with the climate change lead to further deterioration of the situation. The ingestion of mycotoxins causes several health issues leading in the worst case to cancer in humans and animals. Common intervention strategies against mycotoxin producing fungi, such as the application of fungicides, may result in undesirable residues and in some cases to a stress induction of mycotoxin biosynthesis. Moreover, development of fungicide resistances has greatly impacted pre- and postharvest fungal diseases. Hence there is the need to develop alternative strategies to reduce fungal infestation and thus mycotoxin contamination in the food chain. Such a strategy for natural competition of important plant-pathogenic and mycotoxin producing fungi could be *Trichoderma harzianum*, a mycoparasitic fungus. Especially in direct comparison to certain tested fungicides, the inhibition of different tested fungal species by *T. harzianum* was comparable, more sustainable and in some cases more effective, too. Besides substantially reduced growth rates, a transcriptional based inhibition of mycotoxin biosynthesis in the competed *Aspergillus* species could be shown. Furthermore it could be clearly observed by high-resolution Scanning Electron Microscopy (SEM) that *T. harzianum* actively attaches to the competitor species followed by subsequent enzymatic lysis of those mycelial filaments. The analyzed isolate of *T. harzianum* MRI349 is not known to produce mycotoxins. In this study it could be successfully proven that *T. harzianum* as a biological competitor is an effective complement to the use of fungicides.

1. Introduction

Plants and plant derived foods are exposed to a strong increase in fungal infestations over the last decades and thus are prone to contaminations by mycotoxins. This fact is not only reasoned by weather extremes associated with the climate change, but also by rising fungicide resistances which greatly impact pre- and postharvest fungal diseases (Brent and Hollomon, 1998; Georgopoulos and Skylakakis, 1986). Among human-, animal- and plant pathogens, filamentous fungi in general have a high risk of becoming resistant to fungicides. Their intrinsic potential to effectively adapt to certain growth conditions using their biosynthesis machinery able to produce plenty of biological active secondary metabolites (Rohlfs et al., 2007) may support this fact. Furthermore the selection pressure acting on individual spores of highly variable spore conglomerates as a result of mating, sexual reproduction and recombination (Kendall and Hollomon, 1998) may be favored especially under harsh growth conditions. In pre- and postharvest management of plant derived foods, the risk of development of fungicide resistance in important food-related fungal species is additionally exacerbated by the year-round availability of susceptible host tissues. Another point which supports fungal infestation and resilience is the fact, that food commodities are stored almost continuously after harvest under often suboptimal conditions. Important mycotoxin producing fungal species which affect plants before and after harvest belong mostly to the species *Aspergillus*, *Alternaria* and *Fusarium*. Examples are *Aspergillus flavus*, *Aspergillus carbonarius* and *Alternaria alternata* occurring on different food commodities and *Fusarium oxysporum* occurring on different vegetables, fruits and crops. These fungi are all able to produce important mycotoxins such aflatoxin, ochratoxin, alternariol or trichothecenes. Various parameters could have an impact on the infestation of plants by fungi and the subsequent formation of mycotoxins. These are the susceptibility of the host-plant, composition and quality of the soil, temperature, humidity, pH, insects as well as the plant- and soil associated microbiome. Moreover, conditions which impose stress to the fungus such as substances with oxidative activity, e.g. reactive oxygen species which are produced by plants as defense reaction (oxidative burst), or by the action of fungicides or preservatives (Jayashree and Subramanyam, 2000; Schmidt-Heydt et al.,

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2013), could induce mycotoxin biosynthesis while reducing in parallel the growth of the fungus. This lead to delusive estimations about the toxin content (Jurado et al., 2008). Furthermore, it is well-known that fungi may adapt their secondary metabolite profile depending on the environmental conditions (Battilani et al., 2016; Magan et al., 2011; Medina et al., 2015), making them less susceptible for sustainable intervention strategies. In contrast, influencing factors promoting the vitality of the plant and thus strengthening their defense reaction against fungal infections could be used to substantially reduce mycotoxin production both on the field and during storage (Mayer et al., 2014). Various chemical and biological based antifungals are available on the market to reduce fungal infections on pre- or postharvest plants (Atehnkeng et al., 2016; Marechera and Ndwiga, 2015; Nene and Thapliyal, 1993; Thomson, 1973). In this study different fungicides, in comparison to the use of the mycoparasitic *Trichoderma harzianum* BFE349, have been analyzed in their ability to inhibit the growth and thus mycotoxin biosynthesis of important food related fungal species.

2. Materials and methods

2.1. Fungal strains and growth conditions

Fusarium oxysporum MRI271, *Alternaria alternata* MRI001, *Trichoderma harzianum* MRI349, *Aspergillus carbonarius* MRI13 and *Aspergillus flavus* MRI19 from the culture collection of the Max Rubner-Institut have been used throughout the experiments. Additionally, but only for the fungicide experiments, the non-mycotoxin producing species *Verticillium dahliae* and the ochratoxin producing typical postharvest species *Penicillium verrucosum* BFE808 were used. A 200 µl spore solution of each species containing 10^5 spores per ml was mixed in a Tween-80/NaCl-mixture (20%). In case of the fungicide experiments the spore solution was then plated onto a 20 cm Petri-dish (Greiner Biolabs) containing YES medium (yeast extract 20 g/l; sucrose 150 g/l; agar 20 g/l). In the competition experiments both, the spore suspension of *Trichoderma harzianum* MRI349 as fungal biocide and the spore suspensions of the competitor species were applicated as single point inoculation each 100 µl on half a plate. All experiments have been incubated for 7 days at 25 °C in biological triplicates.

2.2. Fungicides and application

Fungicide dilutions were composed using sterile Milli-Q-water as described in Table 1.

A cellulose platelet containing 90 µl of the respective fungicide dilution was placed in the middle of each Petri dish containing the plated spore suspension of the fungus which should be inhibited. A cellulose platelet with the respective amount of sterile Milli-Q-water was used as negative control. The fungal cultures were then incubated at 25 °C for 7 days respectively 29 days in the dark. Afterwards the inhibition zone around the fungicide containing platelet was measured. All experiments have been done in biological triplicates.

Table 1
Applied fungicide concentrations.

Fungicide	Applied concentration
Mancozeb (dithiocarbamate)	0.68 g/100 ml
Azoxystrobin (pyrimidin derivative, strobilurin)	0.12 g/100 ml
Difenoconazol (mixture of 2 triazol derivatives)	0.06 g/100 ml and 0.08 g/100 ml
Iprodione (imidazol derivative)	0.32 g/100 ml
Pyraclostrobin (strobilurin)	0.02 g/100 ml

2.3. Growth assessment

For analyzing growth and morphology the diameters of the inhibition zone around the fungicide containing platelet were measured two times, rectangular to each other and the mean value was calculated.

2.4. Quantification of ochratoxin-A/B and aflatoxin by HPLC

For determination of ochratoxin and aflatoxin B_1 biosynthesis, an agar plug (Ø 1 cm, 250 mg) of the respective colony (*Aspergillus flavus* or *Aspergillus carbonarius*) was taken from the region between center and edge of the colony with the aid of a sterile corer. This agar plug with the adhering mycelium was transferred into 2 ml reaction tubes, and 1 ml of chloroform was added. The fungal mycelia were extracted for 30 min at room temperature on a rotary shaker (Eppendorf, Germany); the mycelia were discarded and the chloroform extract was evaporated to dryness in a vacuum concentrator (Savant Instruments, USA). The quantitative determination of ochratoxin A and aflatoxin B_1 has been performed as described by Sato et al. (2010) on a VWR-Hitachi Chromaster DAD/UV–Vis 600 quaternary LPG System equipped with an auto-injector, column oven and fluorescence detector. The limit of quantification was 25 pg (on column). Data collection and handling was done with the Software package EZE 3.3.2. All standards were obtained from Sigma (Taufkirchen, Germany) with a purity of ≥ 99%. The mean value of three independent replicates has been used for statistical calculation.

2.5. Visualization of the fungal interaction with (cryo)-scanning electron microscopy (cryo)-SEM

For studying the interaction of *Trichoderma harzianum* with the different mycotoxin producing fungi cryo-SEM was chosen as the preferred analysis method, because it allows the in-situ investigation of the delicate and fragile fungal hyphae network and conidiophores in their frozen natural hydrated state (Alves et al., 2013).

For the first step of cryo-fixation an agar square of approximately 7 × 15 mm was sliced with a scalpel from the plates with 7-day-old grown cultures, fixed on a sample transfer shuttle with conductive mounting medium (1:1 mix of Tissue-Tek® O.C.T™ compound and colloidal graphite, Agar Scientific Ltd., Stansted, United Kingdom) and quickly frozen by plunging in nitrogen slush (ca. −210 °C). After being transferred to the cryo preparation chamber (PP2000 T, Quorum Technologies Ltd., Laughton, United Kingdom, T = −135 °C), the residual ice contamination on the sample surface was removed by sublimation at −90 °C for 15 min. Finally, the sample was sputtered with platinum in Argon atmosphere (60 s coating at 5–10 mA current) to prevent charging effects during SEM analysis and transferred to the chamber (T = −135 °C) of the SEM (Quanta 250 FEG field emission scanning electron microscope, FEI, Brno, Czech Republic). The imaging was carried out with an Everhart-Thornley detector under high vacuum conditions ($\sim 3 \cdot 10^{-7}$ mbar), a working distance of 5 mm and an acceleration voltage of 5 kV. In order to differentiate visually the two competing fungal species, chosen SEM images were false-colored using *Adobe Photoshop CC* (version 2015.1.2).

In the case of *Aspergillus flavus* the samples were chemically fixed prior to classical SEM analysis, because work with living samples of this strain is restricted to biosafety level 2 and can therefore not be performed without fixation in the SEM chamber. For this purpose a small piece of each fungal sample was fixed with 2,5% glutaraldehyde in 0,1 M sodium phosphate buffer (pH 7,4) in a 12-well cell culture plate (Costar®, USA) for 2 h at room temperature (one sample per well). The fixed specimens were washed once with the same buffer and twice with Milli-Q water (Milli-Q Advantage A10 Water Purification System, Millipore, Germany) and dehydrated with ascending concentrations of ethanol (25%, 50%, 75%, 90%, 95% and twice 100%). Each incubation step was carried out for 30 min at room temperature; as an exception,

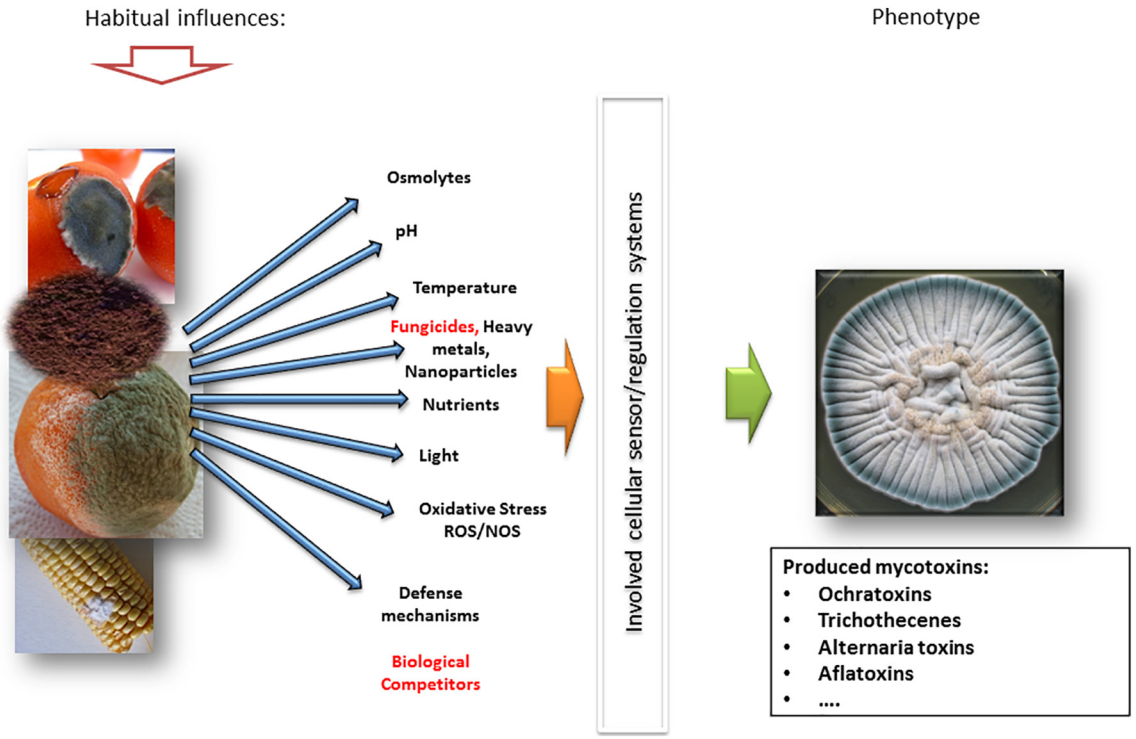

Fig. 1. Scheme of habitual influences which may affect fungal growth and mycotoxin biosynthesis.

the 90%-step was carried out over night at 4 °C. For drying critical point drying in liquid CO_2 was applied (BAL-TEC CPD 030 Critical Point Dryer, BAL-TEC AG, Liechtenstein) and the samples were prepared for SEM analysis by mounting them on aluminum stubs (Ø = 12 mm, Agar Scientific, UK) with conductive carbon cement (Leit-C, Plano, Germany) and sputter-coating with platinum for 60 s (Q150T ES, Quorum Technologies Ltd., UK). SEM observations were carried out under the same conditions as described above.

3. Results

Comprehensive knowledge about the physiological mechanisms behind the regulation of growth and mycotoxin biosynthesis of filamentous fungi is the prerequisite for the development of sustainable prevention strategies against fungal infestations in order to prevent rejection of contaminated harvests due to exceeding the allowed mycotoxin treshold (Fig. 1). The application of fungicides is cost-intensive and sometimes negotiated because of possibly remaining residues in the food matrix or within the soil. Besides, such residues could have deleterious effects on the soil associated microbiome and fungicides are static substances to which a fungal contaminant may be able to adapt. Thus, a living organism as biological competitor may be more successful due to its ability to react on the adaptive response of the mycotoxin producing fungus which should be competed. Consequently, biological alternatives should be favored on long-sight or kept as complementary strategy to reduce the necessary amount of fungicides. In this study *Trichoderma harzianum* has been applied comparatively to different commercial fungicides to inhibit mycotoxin producing fungi. Furthermore, using high resolution SEM the mechanistic action of *Trichoderma* competition has been investigated.

Comparing the fungicides Mancozeb, Azoxystrobin, Difenoconazol, Iprodion and Pyraclostrobin, it could be clearly seen (Fig. 2A), that the former was most effective in inhibition of *Verticillium dahliae*, a main causative of *Verticillium* rot commonly described on different soil-borne vegetables (Babadoost et al., 2004). Also substantially reduced in growth but to a lesser extent was *Penicillium verrucosum* a wheat contaminating ochratoxin and citrinin producing post-harvest fungus and *Alternaria alternata* which occurs on different vegetables such as tomatoes and carrots. At least both, the plant pathogenic fungus *Fusarium oxysporum* and *Aspergillus carbonarius*, occurring on grapes, showed only a weak inhibition in their growth rate. The other applied fungicides were less effective regarding the inhibition of fungal growth in this experimental setup. Furthermore, in order to analyze if the fungicides have either a longer-lasting inhibitory action on fungal growth or if the fungi were able to start to grow after a period of retardation or if resistant spores survive the procedure, the zone of inhibition around the fungicide-containing platelet was reassessed again after prolonged incubation time of 29 days of growth (Fig. 2B). After this time span only *Verticillium dahliae*, *Penicillium verrucosum* and to a lesser extend *Aspergillus carbonarius* showed substantial signs of inhibition, especially by the use of the fungicide Mancozeb. In case of *Aspergillus carbonarius* (Fig. 2C) it could be exemplarily visualized that after prolonged incubation the fungicide largely loses its effectivity and the fungus overgrows the region with the fungicide containing platelet. In contrast *Trichoderma harzianum* MRI349 in direct competition to the respective mycotoxin producing fungi was able to overgrow the mycotoxin producing species (control, Fig. 3A) in varying degrees from *Aspergillus flavus* and *Aspergillus carbonarius* to *Alternaria alternata* and *Fusarium oxysporum* (competition, Fig. 3B). Obviously, depicted on the SEM images (Fig. 3C) which were made from the region were both species have contact to each other, the filaments of *Trichoderma harzianum* (with green coloration) actively adhere to mycelia, conidiophores and spores of the competitor strain. This mechanism could be clearly observed especially in case of *Aspergillus carbonarius* and *Fusarium oxysporum*. Interestingly the attacking sticky filaments of *Trichoderma harzianum* collapse simultaneously while attaching to the competed species. Both, *Trichoderma* and the attacked species show clear signs of lysis and disintegration due to the action of excreted lyzing enzymes such as chitinases. Strikingly, *Aspergillus flavus*, growing very close to the substrate forming extensive substrate mycelia besides aerial mycelia with conidiophores, was less susceptible for an attack by *Trichoderma* hyphae compared to the other species. In the next step we

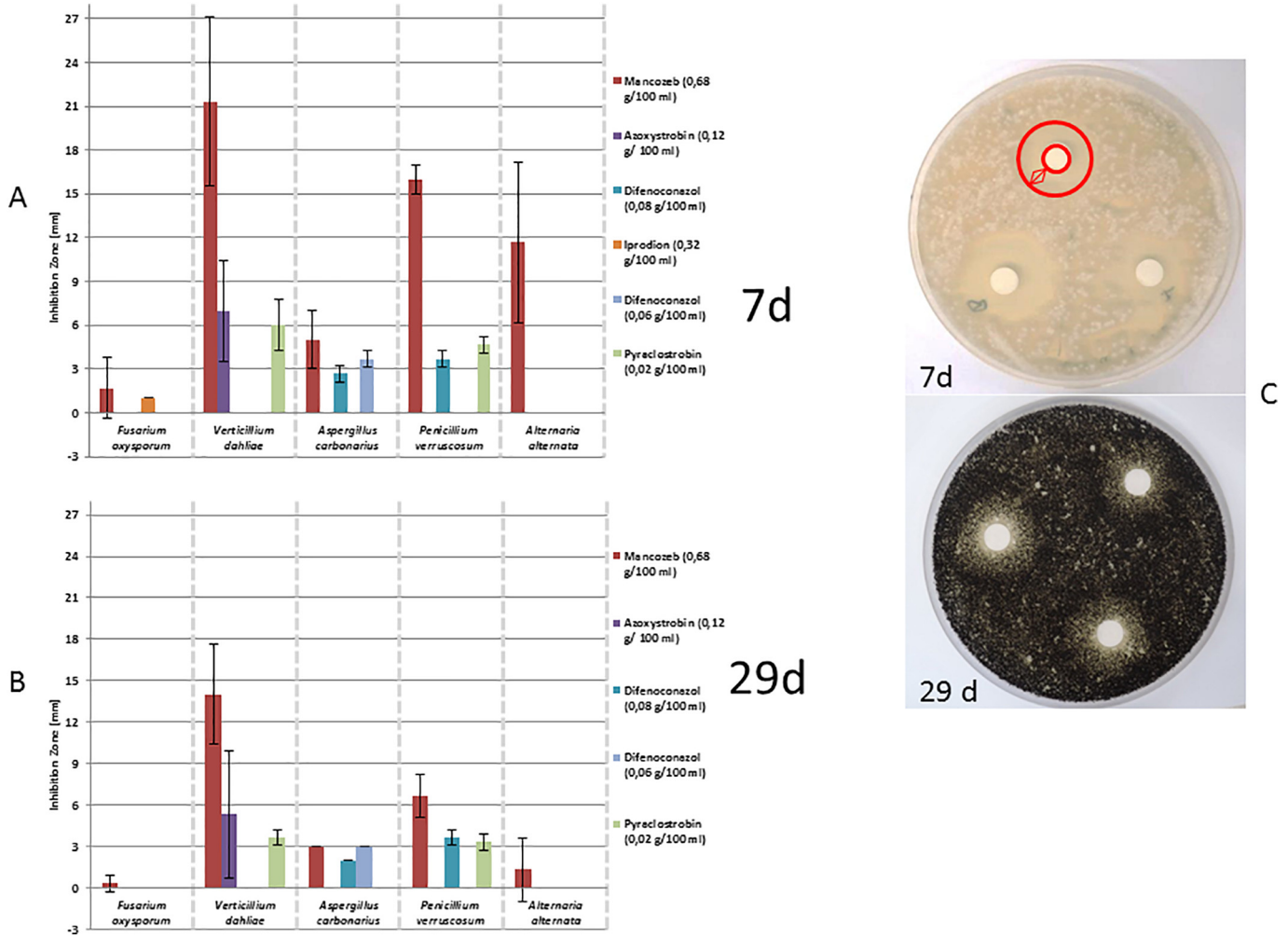

Fig. 2. Fungicide inhibition test: Quantitative assessment of the inhibition zone around the fungicide containing cellulose platelets after 7 days of growth on YES medium in the dark at 25 °C (A) and after prolonged incubation of 29 days (B). C shows the visualization of the diameter of the inhibition zone after 67 days of growth surrounding the cellulose platelets containing the respective fungicide solution (picture above) and the development of resistance against the applied fungicides after prolonged incubation of 29 days, exemplarily in case of *A. carbonarius* (picture beyond).

were interested in the mycotoxin production by the competed species under competition. Since mycotoxin production is an important health issue in context to fungal infestation, it is of utmost importance if the mycotoxin producing fungi show a (stress-) induction of mycotoxin biosynthesis under competition with *Trichoderma* as it has been described by the action of some fungicides (Ellner, 2005; Schmidt-Heydt et al., 2013). This was actually not the case, shown on Fig. 4. Both, the production of ochratoxin by *Aspergillus carbonarius* and aflatoxin B_1 by *Aspergillus flavus* were substantially reduced in parallel to growth reduction and proportional to the distance between *Trichoderma harzianum* and competed species after 7 days of competition.

4. Discussion

The development of alternative and sustainable prevention strategies, especially low-cost applications for less-developed countries should be forwarded to inhibit fungal infestation and thus mycotoxin contamination in food and feed. Special emphasis should be taken to avoid residues and unwanted side-effects such as the physiological reaction of fungi to compete stress, presumably induced by the action of substances that result in the upregulation of toxic secondary metabolites. Such cases have been described for the induction of the mycotoxin aflatoxin B_1 in *A. flavus* (Jayashree and Subramanyam, 2000) and for citrinin and ochratoxin in *P. verrucosum* (Schmidt-Heydt et al., 2013) by the action of specific fungicides. Similar effects have been shown by Ellner (2005) for fungicides based on Strobilurins if they were applied on the field at suboptimal time points. Obviously this effect may be observed for other fungicides, too (Nakajima, 2010). Complementary strategies based on biological competitors should therefore be favored on long-sight. In this study it could be clearly shown by high-resolution Cryo-Scanning Electron Microscopy (Cryo-SEM), that *Trichoderma harzianum* MRI349 as mycoparasite not simply overgrow the competitor species but actively adhere to the mycelial filaments of the attacked fungus followed by cell lysis, penetration and disintegration of mycelia and conidiophores. This strong mycoparasitic activity against the tested mycotoxin producing fungi analyzed throughout this study is supported by the excretion of lyzing enzymes such as chitinases, laccases, serine proteases and further cell-wall-degrading enzymes (Chet et al., 1981; Harman et al., 1993; Steyaert et al., 2003). Various biological competitors are in use against soil-borne pathogens (Haas and Défago, 2005; Woo et al., 2006). Atoxigenic isolates of usually mycotoxin producing fungal species are applied against harmful species producing mycotoxins (Atehnkeng et al., 2016). This system works well, but there remains the undeniable risk that the atoxigenic strain revert or transform into a toxigenic one, e.g. by molecular mechanisms such as horizontal gene transfer of an intact toxin gene cluster. Such events occur in nature and there is an increase in publications which describe such mechanisms especially for fungi living in close contact within the same habitat

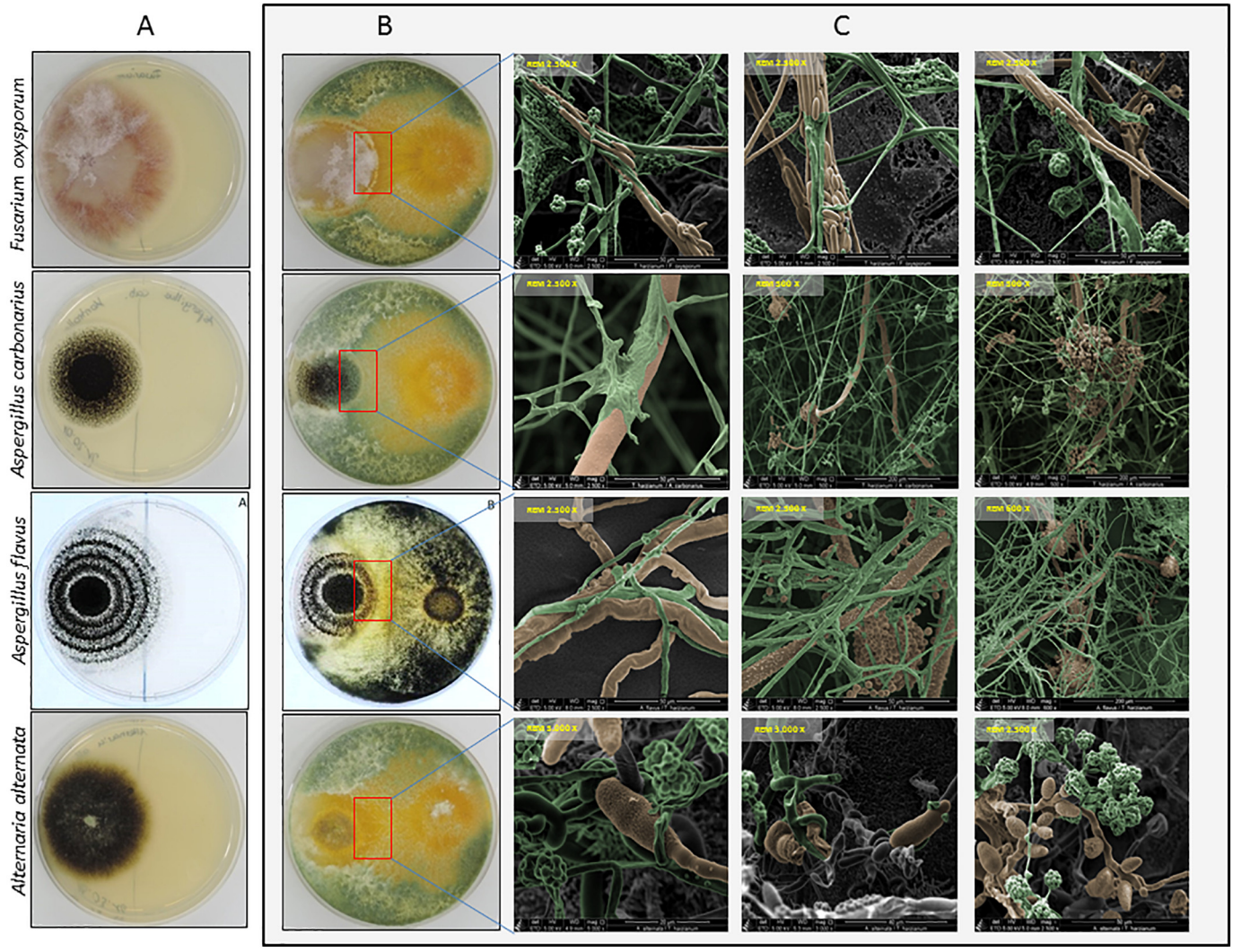

Fig. 3. Phenotypical growth on YES medium after 7 days at 25 °C of either the fungal species alone (A) or under competition with *T. harzianum* (B). *Trichoderma* overgrows the mycotoxin producing fungus with different effectivity. SEM pictures (C), made from the region were both species have contact to each other. Filaments of the attacked fungi show clear signs of lysis as a result of excreted *Trichoderma* lyzing enzymes such as chitinases. The post-colored SEM images present *T. harzianum* in green and the competing species in light brown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Cheeseman et al., 2014; Fitzpatrick, 2012; Rosewich and Kistler, 2000; Schmidt-Heydt and Geisen, 2017; Walton, 2000). Furthermore, the often highly conserved structural composition of secondary metabolite clusters, such as it has been described for the citrinin biosynthesis gene cluster (Stoll et al., 2016), implicates such events, too. Another point is that atoxigenic strains may produce further harmful mycotoxins, such as the cyclopiazonic acid production by non-aflatoxigenic strains of *A. flavus*, and thus cause still health problems if ingested by humans and animals (Chalivendra et al., 2017). *Trichoderma harzianum* as mycoparasitic soil fungus may be an alternative use. This species grows very well and produces an arsenal of enzymes to actively compete and kill plant-pathogenic and mycotoxin producing fungi occurring in concurrence within the soil (Lorito et al., 1996; Saravanakumar et al., 2017). Another positive effect called “priming”, is the induction of acquired resistance against plant-pathogens (Altomare et al., 1999; Elad, 2000) triggered e.g. by plant resistance proteins and salicylate-, jasmonate- signaling pathways. These pathways are induced in plant tissue upon plant-fungus interactions. Furthermore, the soil could receive a gain in nutritional value due to enzymatic degradation of fungal biomass by *Trichoderma* lyzing enzymes. Last but not least it is improbable that *Trichoderma harzianum* will receive a toxin gene cluster from a mycotoxin producing species. The reason for this assumption is the fact, that a non-toxigenic isolate with e.g. a deletion in a single gene of a mycotoxin gene cluster has the “evolutive pressure” to complement the biosynthesis cluster as the production of the coded metabolite may have advantages for this species to survive on the habitat. Of course, non-toxic species developed alternative adaption strategies as in the case of *Trichoderma harzianum*, eating other fungi. The strain *T. harzianum* MRI349 has actually been sequenced by us and carries no gene clusters of known mycotoxins within its genome.

5. Conclusion

Natural competitors such as the mycoparasitic *Trichoderma harzianum* may serve as safe and effective complementary strategy for reducing fungal diseases.

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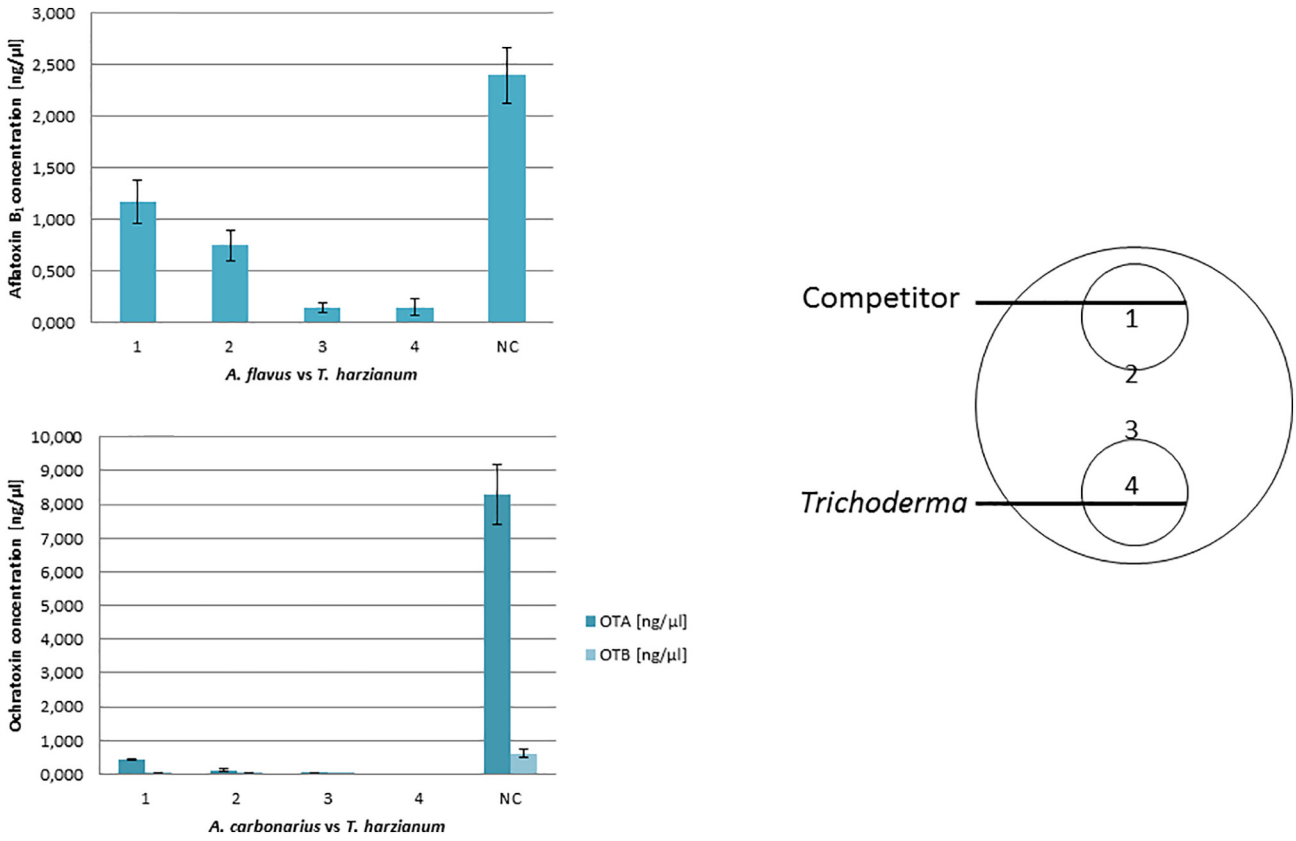

Fig. 4. HPLC quantification of the mycotoxins ochratoxin A/B produced by *A. carbonarius*; aflatoxin B_1 produced by *A. flavus* grown in competition with *T. harzianum*. The bars present the mean values of three biological replicates for 4 different sampling points in comparison to a negative control (NC, grown without *T. harzianum*) after 7 days. The visualization on the right side shows how the fungi were applied on the agar plates and assigns the accumulated toxin with respect to the distance to *T. harzianum* (*at four different sampling points*).

Rubner-Institut and the University of Weihenstephan-Triesdorf.

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